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Alternative, indirect measures of ballast water treatment efficacy during a shipboard trial: a case study

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A shipboard study was conducted aboard the cruise ship *Coral Princess* during a scheduled cruise from San Pedro, CA, USA to Vancouver, British Columbia, Canada. The investigation involved three members of the global TestNet group, with experience in certification testing of ballast water treatment systems (BWTS) designed to eliminate entrained invasive species. A UV-based ballast water treatment system had been employed aboard the vessel for more than 10 years. A variety of established and experimental assessment techniques were employed, both aboard the ship and following shipment of samples via road (5 days) and air (7 days) to remote laboratories. The study was designed to compare the performance of different techniques in assessing BWTS compliance with international regulations, and to test the feasibility of compliance assessment by Port State Control internationally using different laboratories. Overall, biological end-points showed effective treatment of ballast water as judged by the percentage removal (mortality) of organisms in treated samples. Sample transport indicated generally good potential for 'off-site' sample analysis and displayed a possible latent effect of treatment as judged by a decline in photosynthetic yield associated with delayed analysis.

Introduction

Ten years after the adoption of the International Convention for the Control of Ballast Water and Sediments, full ratification of the convention has yet to be achieved. Outstanding issues include uncertainty over how the convention will be enforced following its entry into force. Principal sticking points relate to ambiguities over biological standards that form the basis for both IMO D2 regulations and US federal regulations, with consequences for ballast water sampling and analytical protocols. Differences of interpretation exist among test centres responsible for certification testing. These are primarily related to the size-based categorization of planktonic organisms, the sampling effort required to characterize precise numeric standards and the analytical protocols needed to achieve a universal definition of viability that satisfies the 'live/dead' threshold on which the regulations are based. Resolution of these obstacles is important in allowing a smooth transition to post-ratification status and establishing much-needed confidence in an environmental technology market that requires clear goals, performance standards and a uniform compliance doctrine.

While sized-based standards based on live organism counts cannot be changed ahead of full ratification, an eventual move to more integrated Ballast Water Treatment System (BWTS) performance measures less reliant on individual organism counts may be anticipated. However, analytical techniques that can be calibrated directly or indirectly against current numeric standards will be of particular interest. It is important that these methods combine rigour and precision in order to withstand a potential legal challenge, in the likelihood that rigorous numeric standards will remain a component of future ballast water regulations. Several reviews have been made of candidate analytical protocols that might comprise an international suite of assessment tools (Gollasch and David 2010; Jørgensen et al. 2010; MEPC 62/INF31 2011). Although a 'one-size-fits-all' approach is unlikely, Wright and Welschmeyer (Wright and Welschmeyer 2015) among others have advocated the inclusion of one or more benchmark 'common denominator(s)' as part of a universal approach to compliance assessment. Such an initiative will require collaboration among the centres involved with certification and compliance testing worldwide. Of particular interest will be comparative live staining techniques and the linkage of more indicative, integrated measures to current numeric standards.

The objective of the current study was to assemble a suite of biological efficacy tests, both prescribed and alternative, and to execute the test protocols by three independent test organizations on a single source of shipboard 'control and treatment' ballast test water. Some of the tests adhered to specific requirements of existing ballast water testing protocols (e.g. US EPA/ETV 2011), and other tests were selected as alternative methods, with considerable emphasis on rapid, simple methodologies. The critical

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feature of the experiment involved the logistics of sampling/analysis. The study was conducted in May 2013 aboard the Princess Cruise Lines vessel, *Coral Princess* during the last leg of the vessel's transition cruise from its winter base in Fort Lauderdale to its summer base in Vancouver, Canada. The study involved members of the Global TestNet group, Environmental Research Services (ERS), Baltimore, MD, USA, Moss Landing Marine Laboratory, Moss Landing, CA, USA and the Netherlands Institute for Sea Research, Texel, Netherlands. Ballast water was acquired and treated in the last port of call San Pedro, CA with attendant assays executed aboard vessel while en route to Canada. In addition to shipboard testing by ERS two small aliquots of the same water were delivered by air and ground service to Europe (Netherlands Sea Research Institute, NIOZ) and US-California (Moss Landing Marine Laboratories, MLML), respectively. This case study addressed the question: 'Can three independent laboratories achieve consistent results for ballast water treatment assessment using on-site and off-site sample analyses?'

Materials and methods

A Hyde Marine 'Guardian' BWTS had been installed aboard the vessel in 2003. The system consists of a primary disk filter mounted in series with a UV irradiation unit, and had been in continual operation without modification since then.

Sampling

Two pairs of ballast tanks, 5P/5S and 6P/6S were selected for the trial. By prior agreement these pairs of tanks were emptied before reaching Californian waters and the ship arrived at San Pedro with these tanks empty. During ballast water uplift, tanks 5P/S and 6P/S were filled sequentially with treated ballast water. The sampling team took and processed samples from a sampling port located upstream from the BWTS with water passing through both components of the system (filter + UV). In this case, water was sampled before it reached the BWTS and these samples therefore represented untreated 'challenge water'. The sampling system consisted of a 4 cm hose directed sequentially to each of four 180 L tubs. Water was filtered through 35 µm mesh plankton nets (50 µm diagonal mesh size). During filtration, each net was submerged under the surface of the water in each tub in order to soften the impact of the filtration procedure on the planktonic organisms. The plastic bottle that formed the 'cod-end' of the net also had 35 µm mesh 'windows' to facilitate the filtration process. As tubs were filled sequentially, each received similar, or in some cases identical, volumes of water during the sampling process. After 5P&5S tanks had been filled, nets were rinsed into 500 ml. plastic bottles. After tanks 5P and 5S were full, the same sampling procedure was employed while tanks 6P and 6S were being filled. During the sampling procedure, as each tub was filled, filtrate was removed by sump pumps, one per tub, to an empty ballast tank (No. 2) that served as a 'slop' tank, thereby preventing filtered water from disposal in the ship's bilge.

Sampling of challenge water commenced at 1330 h and was completed at 1545 h on 19 May. The total volume of challenge water processed was 5.00 m^3. As the vessel approached Vancouver 40 h later (21 May), treated water samples were collected from a sampling port located downstream from the BWTS in the same sequence as uplift but after passing the water only through the UV system (bypassing the filter). Thus, the final treatment consisted of filtration and UV irradiation at ballast uplift, and UV irradiation at discharge. A total of 8.2 m^3 of treated water was processed in this manner. Sample analysis began aboard the ship and continued at a nearby hotel. Selected split water samples ('challenge' and treated) were shipped by road to MLML and by air in a B-box (www.ballastwaterbox.nl) to NIOZ for further analysis.

Analytical methodology

ATP

Adenosine tri-phosphate (ATP) was determined on GF/F 25 mm filters (0.7 µm nominal pore size) using the method detailed by Karl (1980) and incorporated in the international standard operating procedures of the Joint Global Ocean Flux Study (JGOFS). Five-minute extractions were made with boiling 2.5mM Tris buffer (4 mL per sample), and extracts were kept frozen (− 20.0°C) until analysis on a Turner Designs 20/20 luminometer using Promega Inc. Enlightem™ Luciferin-luciferase enzyme preparations and ATP standards. At MLML samples were filtered, extracted and kept frozen until the day of analysis.

> 50 µm size category

Although the primary focus of this study was on the 10–50 µm size fraction comprising mostly phytoplankton and protists, live counts of the > 50 µm (zooplankton) were also recorded. Counts were made of live organisms in challenge and treated water samples commencing within 2 h of sample collection. Motility was the principal criterion used to define viability, although neutral red provided live/dead confirmation for non-motile forms. The higher concentration of organisms in challenge water necessitated counting sub-samples, each involving several hundred organisms from at least five different taxonomic groups. In view of the small numbers of live organisms present in treated samples, it was necessary to count the whole sample after appropriate concentration by filtering through a 35 µm net.

10–50 µm size category

Numeric counts. Numeric counts of live organisms in the 10–50 µm size range were made by epifluorescence

microscopy following staining by fluorescein diacetate (FDA)/*chloromethyl*-fluorescein diacetate (Steinberg et al. 2011) or FDA alone as well as flow cytometry (FCM).

Measurement of growth potential. Most determinations of reproductive or growth potential require some incubation time for this potential to be realized. During this trial, challenge water and treated water samples were subjected to two different periods of incubation. The first incubation period was initiated by the shipboard sampling team immediately following sample collection. Phytoplankton grow-out was determined by exposing challenge and treated water samples to a 48 h grow-out period under nutrient-rich conditions. Nutrients were added to each pooled sample as F/2 growth medium (Guillard 1975). Samples were placed in a temperature-controlled incubator under continuous fluorescent growth light illumination (40 µM photons m^{-2} s^{-1}) at 15°C, the ambient water temperature at discharge. Measured end-points comprised chlorophyll *a* determination using a Turner Designs Aquafluor fluorometer, Model # 8000-001 calibrated using a solid standard at the University of Maryland Chesapeake Biological Laboratory analytical services division. Following incubation the chlorophyll a fluorescence was re-measured and the percentage change in chlorophyll *a* calculated.

Concomitant measurements were also made of photosynthetic yield using a Turner Designs 'Ballast Check' fluorometer read following a dark adaptation period. Photosynthetic yield provides an indication of the ability of photosynthesizing cells to convert solar energy into fixed carbon. As such, it measures the capacity for photoautotrophic growth as an increase in fluorescence from dark-adapted minimal fluorescence (Fo) to maximum fluorescence (Fm) under saturating light. Under nutrient-rich conditions Fv/Fm in healthy cells are in the 'high range', 0.5–0.7. Photosynthetic yield in challenge and treated water samples were determined before and after the same 48 h incubation regime as used for chlorophyll *a* measurements. At both remote laboratories (MLML and NIOZ), photosynthetic yield was also measured using a Walz PAM fluorometer.

Taxonomic counts

Samples of untreated and treated water were examined microscopically using a Fisher stereoscope and an Olympus BH2 epifluorescence scope. Counts of intact cells were made using a Sedgwick Rafter cell. Taxonomic identification was initiated on board and was continued over the succeeding weeks on samples preserved in Lugol's solution.

Primary productivity using ^{14}C

The photosynthetic incorporation of inorganic carbon into autotrophic biomass by phytoplankton was measured by the ^{14}C technique; the technique provides unambiguous, quantitative assessment of the potential inactivation of photoautotrophic metabolism resulting from ballast water treatment protocol. Procedures for ^{14}C preparation and analysis followed that of Welschmeyer et al. (1993). Incubations were for 24 h, in a controlled-temperature incubator (^{13}C), under continuous, weak light (ca. 25 µM photons $m^{-2}s^{-1}$). Incubation bottles (160 mL) for both untreated uptake water and treated discharge water were placed on a rotating plankton wheel to keep cells in suspension and to ensure equal average irradiance values for all samples throughout the incubation.

Results

> 50 µm size category (zooplankton)

These data show that the zooplankton concentrations in water at pick-up met requisite US Coast Guard and IMO shipboard test criteria for challenge water, and were similar to challenge water zooplankton concentrations recorded from the 2008 Alaska Shipboard Technology Evaluation Program (STEP) trial 2 (Wright 2009). Results indicated a mean concentration of 11.02/m^3 live organisms in the discharge water, representing a 99.92% removal of this size

Table 1. Numeric counts of live > 50 µm size category (zooplankton) in challenge and treated water.

Challenge water samples, San Pedro, 19 May 1500–1700 h			
Sample 1 collected during filling 5P&S; Net A	Sample 2 collected during filling 5P&S Net B	Sample 3 collected during filling 6P&S Nets A & B	Mean concentration density of Challenge Water samples ± SD of samples 1–3
10,953/m^3	16,127/m^3	13,299/m^3	13,460 ± 2,591/m^3

Treated water samples, off Washington State coast, 21 May 0830–1100 h.				
Sample 1 collected during filling 5P&S; Net A	Sample 2 collected during filling 5P&S Net B	Sample 3 collected during filling 6P&S; Net A	Sample 4 collected during filling 6P&S; Net B	Mean concentration of Treated Water samples ± SD of samples 1–4.
7.4/m^3	4.94/m^3	19.7/m^3	12.06	11.02 ± 6.5/m^3

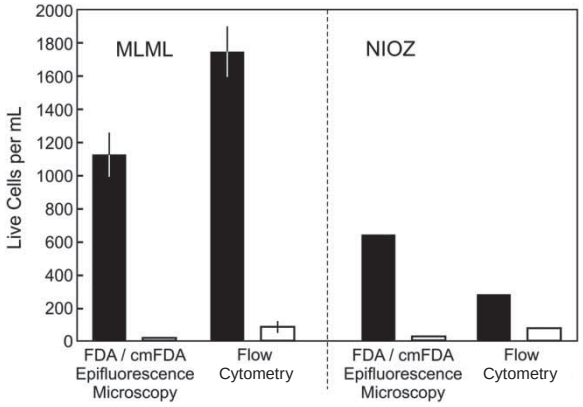

Figure 1. Comparison of live cell counts from 10 to 50 µm size category (phytoplankton/protists) analysed by two different methods (FDA/cmFDA staining + epifluorescence microscopy and FDA staining + flow cytometry) at two different laboratories, 5 days and 7 days, respectively, following shipboard sample collection. (Samples were sent by road to MLML and by air to NIOZ soon after exiting the vessel in coolers over ice packs at $< 10°C$.) Solid bars (on the left) refer to challenge water samples; hollow bars refer to treated water samples.

class. These numbers are within the US Coast Guard STEP requirement of 98% removal and very close to the IMO discharge requirement of < 10 organisms/m^3 for the > 50 µm size class (Table 1).

10–50 µm size category

Numeric counts

Results are shown in Figure 1. Live counts for challenge water samples at MLML were $1.8\times$ higher than those recorded at NIOZ, although mean live counts for treated samples differed by only 23% between these two laboratories. At MLML, FCM resulted in 53% higher live cell counts for challenge water relative to epifluorescence microscopy, although at NIOZ, FCM resulted in challenge water counts 39% lower than epifluorescence microscopy.

Table 2 shows the removal/mortality rates for both techniques for both laboratories. The lower 'removal' rate at NIOZ using FC was due to the lower challenge water counts. At both laboratories FC resulted in higher live counts in treated water samples relative to epifluorescence microscopy. Despite differences in counts of challenge water samples, good agreement between laboratories was found for treated samples, which differed by only 35%.

ATP

ATP determinations in the > 10 µm fraction indicated a 99% decline in the ATP concentration of treated samples relative to corresponding challenge water samples (Figure 2).

Measurement of growth/growth potential ^{14}C Assimilation. While not a routine procedure in ballast water testing

Table 2. Removal percentages of live cells in the 10–50 µm size category (phytoplankton/protists) as determined by two different methods (FDA/cmFDA staining + epifluorescence microscopy and FDA staining + flow cytometry) at two different laboratories, MLML and NIOZ, 5 days and 7 days, respectively, following shipboard sample collection.

10–50 µm size fraction counting technique	MLML	NIOZ
FDA/cmFDA stain + epifluorescence microscopy	98.3	96.2
FDA stain + flow cytometry	94.6	81.6

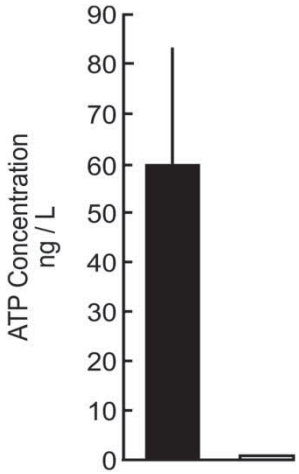

Figure 2. Total biomass as measured by ATP analysis. Solid bar (on the left) refers to challenge water samples; a hollow bar (right) refers to treated water samples.

laboratories, carbon assimilation using ^{14}C has long been employed as a means of assessing primary productivity in the aquatic environment (Peterson 1980). Exposure of the 10–50 µm (phytoplankton) size fraction in uplift and discharge water to $^{14}CO_2$ indicated a 98.1% decrease in ^{14}C assimilation in treated samples relative to challenge water samples under the same conditions (Figure 3).

Chlorophyll a. Chlorophyll *a* concentrations were determined before and after a 48 h grow-out period initiated immediately after taking samples (May 19th for challenge water samples; 21 May for treated samples). As such, incubation was recorded at Day 0, the day of sample collection. Results, shown in Table 3 indicated vigorous growth in challenge water samples subjected to nutrient-rich conditions under lights, a 554% increase in chlorophyll a concentrations over 48 h. Treated samples immediately following collection exhibited a 73% decline in chlorophyll *a* concentration relative to challenge water. This declined a further 71% relative to pre-grow-out conditions, a 92% decline from the original challenge water. Figure 4 shows net chlorophyll growth per day.

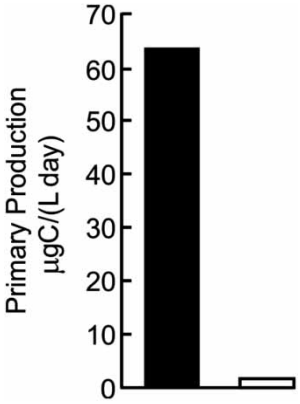

Figure 3. Primary productivity measured as µmC/day as determined by ^{14}C incorporation from CO_2 in challenge water samples (solid bar, left) and treated samples (hollow bar, right).

Table 3. Chlorophyll *a* concentrations (µg/L) before and after grow-out in nutrient-rich conditions under fluorescent light: mean ± SD of three samples.

	Before grow-out	After grow-out
Untreated (challenge) water	16.3 ± 1.35	90.33 ± 0.7
Treated (discharge) water	4.35 ± 0.33	1.28 ± 0.25

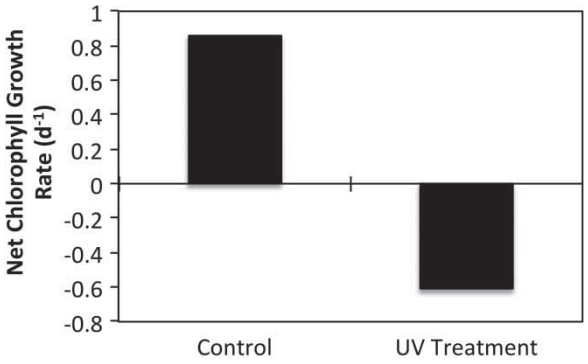

Figure 4. Net chlorophyll growth per day in control (untreated, challenge) water and treated (discharge) water.

A more comparable figure to the foregoing ATP and ^{14}C determinations is a comparison of challenge and treated water samples following grow-out. By this measure treatment resulted in a 98.6% decline in growth potential as determined by chlorophyll *a* (Figure 5).

Photosynthetic yield (Fv/Fm)

Results (Table 4) indicated a high photosynthetic yield in challenge water samples (0.72) that was virtually unchanged (0.71) following a 48 h incubation period under optimal growth conditions. Treated samples at Day 0 showed a 53% decline in yield to 0.348, and a further decline to 0.18 following 48 h exposure to fluorescent light

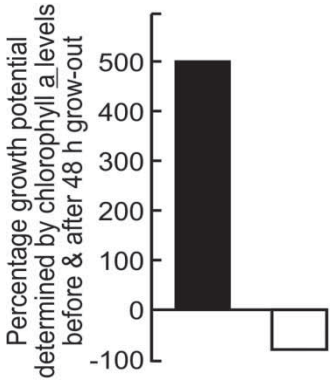

Figure 5. Growth potential in challenge water and treated water measured as chlorophyll *a* concentration before and after grow-out in nutrient-rich conditions under fluorescent light. Solid bar (at left) refers to challenge water samples; hollow bar (right) refers to treated water samples.

Table 4. Photosystem II activity measured as photosynthetic yield in untreated (challenge) water and treated water before and after grow-out in nutrient-rich conditions under fluorescent light: mean ± SD of three samples.

	Before grow-out	After grow-out
Untreated (challenge) water	0.72 ± 0.09	0.71 ± 0.7
Treated (discharge) water	0.35 ± 0.1	0.18 ± 0.025

under nutrient-rich conditions. This represented a 75% decline in photosynthetic yield relative to challenge water.

Other measures of photosynthetic yield were made on reception of challenge and treated water samples at MLML and NIOZ, 5 days and 7 days, respectively, following shipboard collection of treated samples on 21 May 2013. Including the 40 h difference between challenge water sample and treated water sample collection, these laboratory analyses were essentially performed on Day 5 and Day 7 respectively. Variously analysed photosynthetic yield measurements are summarized in Table 5.

Results indicate an overall reduction in photosynthetic yield in treated samples over the period from Day 0 to Day 7, although confounding factors include the different instruments used for the analyses. Both Walz and Turner instruments were employed at NIOZ, where results indicate lower readings for the Walz PAM fluorometer, particularly for treated samples. Yield values recorded for challenge water samples are consistently in the medium to high range, with some evidence of a decline between Day 0 and Day 7. Treated levels appear significantly lower; dramatically so when measured using the Walz fluorometer. Taking into account the continued reduction in yield exhibited by treated Day 0 samples following grow-out there is clear evidence that treatment significantly inhibited photosynthetic activity, although the degree of inhibition

Table 5. Photosynthetic yield (Fv/Fm) determined at intervals following collection of challenge and treated water samples aboard M/V *Coral Princess* 19–21 May 2013.

	Fv/Fm challenge water	Fv/Fm treated water
Day 0 (ERS)	0.72 (Turner Ballast Check)	0.34 (Turner Ballast Check)
Day 5 (MLML)	0.61 (Walz)	0.02 (Walz)
Day 7 (NIOZ)	0.44 (Turner Ballast Check)	0.26 (Turner Ballast Check)
	0.54 (Walz)	0.012 (Walz)

Samples were transported to respective land-based laboratories by road (MLML) and air (NIOZ).

ranges from moderate to severe depending on the analytical instrumentation used.

Discussion and conclusions

This shipboard trial was conducted aboard a cruise ship during the course of the vessel's regular trade and timetable, and as such provides an example of the task facing Port State Control when assessing compliance with the pending IMO Convention for the Management of Ballast Water and Sediments. The three laboratories involved here with sample collection and analysis collectively have more than 40 years of research and analytical experience dealing with issues associated with the IMO Convention, and are part of an analytical consortium of more than 20 laboratories and centres with a specific focus on the analysis of ships' ballast. Many of these analytical laboratories, including the three participating in this trial have been involved with certification testing of BWTSs. The role of certification testing has been to provide the basis for type approval for BWTS. As such, land-based trials often require the test or 'challenge' water to be augmented with substances designed to create physicochemical conditions mandated by IMO D2 of USCG ETV standards. Cultured organisms have also been added to test water to comply with challenge water biological requirements. Shipboard trials, however, are necessarily conducted on ambient water that has not been manipulated and may vary widely in its composition, both chemically and biologically. This reflects the conditions likely to be encountered in harbor-based compliance assessment. Following entry into law of the IMO Ballast Water Convention compliance assessment will need to be made in several hundred ports, perhaps thousands, worldwide. Difficulties to be addressed include the application of analytical protocols, which will provide an accurate and sensitive assessment of ballast water treatment within the duration of a port visit. In this context, the efficacy of treatment will be judged solely against existing standards as no untreated water will be available for comparison. It has been established that full compliance assessment with published standards in every respect will rarely fall within the time interval of an average port visit.

Accordingly, Port State Control will employ a tiered approach to compliance assessment and enforcement, with primary emphasis on the demonstration by ship operators of familiarity with and maintenance of the BWTS and good record keeping (King and Tamburri 2010; Wright 2012). The latter will include a ballast water management plan, good maintenance and usage logs and, most probably, results of indicative tests performed by port state inspectors together with analyses made by ships' crews. The IMO Flag State Implementation Committee has been charged with providing guidance to Port State Control to ensure universal conformity in Convention enforcement. Embodied in their findings is a 2–3-year grace period providing for the development and adoption of analytical and sampling techniques for streamlining the assessment process yet having the sensitivity needed to demonstrate effective ballast water treatment. It is hoped that such protocols will also eventually bridge the gap between indicative testing and full compliance assessment in a timely manner.

The refinement of suitable techniques that work in a variety of conditions and across a broad spectrum of treatment technologies will require the collaboration of several testing centres. Important variables to be considered relate to the inter-laboratory variation that accompanies any collaborative endeavour of this nature, transit time and conditions (e.g. temperature) involved with definitive (as opposed to indicative) analyses. Also of great importance in compliance assessment is the time taken to reach a conclusive end-point. Most Probable Number (MPN) techniques, for example, which form the basis for bacterial assessment against ballast water regulations require many hours to achieve a definitive result, and usually cannot be concluded satisfactorily within the turnaround time of a vessel in port. Recent application of the MPN approach to the 10–50 µm category, nominally phytoplankton, has indicated that the technique has a potential sensitivity greater than FDA tagging, and may be more appropriate viabilty determination for organisms exposed to UV which destroys cells' ability to reproduce (Wright and Welschmeyer 2015).

It is important that, whichever suite of protocols eventually form the basis for compliance assessment, there should be good correlation among the different end-points, and that the sensitivity achieved should enable precise calibration against published regulations. Results of biological end-points relating to the 10–50 µm size category, nominally phytoplankton and protists, are summarized in Table 6.

The biological end-points summarized in Table 6 show effective treatment of ballast water as judged by the percentage removal (mortality) of organisms in treated

Table 6. Summary of biological end-points measured in this shipboard trial.

End-point	State	Day 0 Lab A	Percentage difference between A and B	Day 5 Lab B	Percentage difference between A and B	Day 7 Lab C	Percentage difference between A and B
> 50 µm size category							
Number of live organisms/m^3	A. Challenge water	13,460	99.92	–	–	–	–
	B. Challenge water	11.02	–	–			
10–50 µm size category							
No. of live cells/mL (FDA/cmFDA)	A. Challenge water[a]	–	–	1,128	98.3	624	96.2
	B. Treated water[a]	–	19.5	24			
Photosynthetic yield Fv/Fm	A. Challenge water[b]	0.72	51.4	0.61	96.3	0.46	82.4
	B. Treated water[b]	0.35		0.02	0.08		
Chlorophyll *a*	A. Challenge water[b]	16.3	73.6	–	–	–	–
	B. Treated water[b]	4.3		–	–		
Chlorophyll *a*	A. Challenge water[b]	16.3	92.2	–	–	–	–
	B. Treated water[a]	1.28		–	–		
ATP	A. Challenge water[b]	–	–	60.1	99.1	–	–
	B. Treated water[b]	–	–	0.56	–	–	
Primary production (^{14}C), µg C/(L day)	A. Challenge water[a]	–	–	63.1	98.1	–	–
	B. Treated water[a]	–	–	1.2	–	–	

Note: Dashes indicate a lack of data.
[a]After grow-out/incubation.
[b]Before grow-out.

samples. While the ratio of photosynthetic yield in challenge water vs. treated water is shown here for comparative purposes, the narrow range in PS yield values (> 0.7 is regarded as high, and < 0.2 is regarded as low) makes this measurement unsuitable for this type of comparison. While high percentage differences between challenge water and treated water samples (98.3% and 82.4%) were recorded at the two remote labs after 5 days and 7 days, respectively, probably of greater significance is the decline in PS yield in untreated (challenge water) samples over the 7-day transit time. However, the dramatic decline in PS yield in treated samples over this time period illustrates another potentially significant aspect of these analyses: namely the apparent decline in functionality of treated organisms over a significant latent period. Observations of zooplankton exposed to UV light have shown delays of several hours/days in recording mortality (Wright and Welschmeyer 2015). The influence of inter-laboratory variability cannot be ruled out (Figure 1). Neither can differential readings among different instruments (Table 5). Nevertheless, these are all factors that will have to be considered when developing a unified global approach to compliance assessment.

When compliance assessment is in force analysts will no longer have access to untreated (challenge water) samples as in the current study, and all analyses will be made on treated samples at discharge irrespective of source water location. Samples may comprise water from mixed locations. Analytical protocols will, therefore, need to be effective over a broad range of physicochemical and biological conditions. Sampling and analytical efforts will be constrained by the duration of port visit. It is therefore important that the relatively short analyses that will feature frequently in compliance assessment correlate well with longer bioassays that may better characterize viability but are generally too lengthy for routine testing. Table 6 includes a variety of assays that demonstrate good agreement, yet differ widely in the time taken to reach a result. Microscopic examination of samples for live/viable residuals of eukaryotic organisms in the > 50 µm size range (light microscopy) and 10–50 µm (FDA/cmFDA + epifluorescence microscopy) has the advantage of being directly applicable to published numeric standards for these organisms. However, this requires painstaking effort and specialized expertise to identify live material and differentiate this from artefacts, particularly in view of the enormous variety of biological material likely to be encountered worldwide. MPN techniques for phytoplankton have end-points that equate with numeric standards and the potential for even lower detection limits than epifluorescence microscopy (Wright and Welschmeyer 2015), yet require lengthy grow-out times that are incompatible with the duration of most port visits. Relative mass of chlorophyll *a* following grow-out provides reliable qualitative data on growth potential (Figures 4 and 5, Table 3) but usually require more than 24 h to obtain results. Primary

productivity measurement using ^{14}C incorporation requires a slightly shorter incubation time, and correlates well with other end-points examined here (Table 6) although the involvement of a radioisotope with accompanying restrictions would probably disqualify it as a routine regulatory tool. ATP levels also indicate sharp differences between untreated (challenge) water and treated water (Figure 2), and have the advantage of relatively short incubation time and the potential to define living material in both the > 50 µm and 10–50 µm size fractions, which can be operationally defined by differential filtration. Moreover, the large drop in ATP concentrations for UV-treated samples reported here (Figure 2) suggest that ATP serves as sensitive indicator of successful ballast water treatment under full-scale, shipboard UV treatment doses. These results contrast with those of First and Drake (2014) who showed inconsistent ATP response to UV under laboratory, bench-scale UV dosing levels.

Most end-points reported here provide, at best, qualitative affirmation that published D-2 and US Coast Guard standards are being met or at least approached. Compliance assessment will involve a tiered approach involving analytical techniques of varying complexity and duration. Increased streamlining of such techniques can only result from increased collaboration among the testing facilities involved. This is one of several collaborations in progress. Such joint projects will, in the future, provide the basis for a unified and consistent approach to compliance enforcement of the IMO Convention for the Control of Ballast Water and Sediments.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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